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# *In Vitro* Activity of Fluorescent Dyes against Asexual Blood Stages of *Plasmodium falciparum*

**Fanny Joanny,[a,b] Jana Held,[a,b] and Benjamin Mordmüller[a,b]**

Institut für Tropenmedizin, Eberhard Karls Universität, Tübingen, Germany,[a] and Centre de Recherche Médicale de Lambaréné (CERMEL), Lambaréné, Gabon[b]

**Many successful antimicrobial drugs originate from synthetic dyes. This paper reports the *in vitro* activity of 14 fluorescent dyes against *Plasmodium falciparum*. Five of these dyes (Hoechst 33342, MitoRed, $DiOC_6$, SYTO 9, and rhodamine B) show activity at a low nanomolar concentration against two *P. falciparum* strains in the histidine-rich protein 2 drug sensitivity assay, while toxicity in HeLa cells is low. These dyes may be a starting point for developing new drugs against *P. falciparum*.**

The increasing resistance of *Plasmodium falciparum* to most drugs in clinical use and first reports of reduced sensitivity to artemisinin derivatives, the latest first-line drugs, made the development of new antimalarial compounds a research priority of utmost importance (20). Since existing drugs belong to only a few chemical classes, there is a high probability of the development of cross-resistance, which may shorten the product life of new drugs. In the history of drug discovery, many drugs were derived from synthetic dyes. Indeed, the pharmaceutical industry developed from the dye industry before World War II (22), and the antiplasmodial effect of dyes was shown more than a century ago by Guttman and Ehrlich (10), who cured two malaria patients with methylene blue. They were inspired to use methylene blue as an antimalarial drug after observing that it stains *P. falciparum* particularly well. Later, the antimalarial activity of other dyes such as rhodamine 123 (19), rhodamine B, Janus green (7), and eosin B (17) was discovered.

With this work, we attempt to revive the use of staining properties as a preselection criterion for new antimalarial drugs. The use of dyes has several advantages: they accumulate in the target organism, they are easily screened for, and they belong to a large family of chemical entities not restricted to known pharmacophores. For this proof-of-concept study, we selected 14 different commercially available dyes staining mitochondria (MitoRed, MitoGreen, Daspei, $DiOC_6$, rhodamine 123, rhodamine B, JC1, SYTO 18), nucleic acids (Hoechst 33342, acridine orange, SYBR green I, SYTO 9), or proteins (carboxyfluorescein diacetate [CFDA], CFDA-succinimidyl ester [CFDA-SE]). Dyes were obtained from the following sources. A yeast mitochondrial stain sampler kit containing $DiOC_6$ (CAS no. 53213-82-4), rhodamine B (hexyl ester perchlorate; CAS number not available), MitoGreen (MitoTracker Green FM; CAS no. 201860-17-5), and SYTO 18 (the structure is proprietary) was obtained from Invitrogen. The mitochondrial stains MitoRed (MitoTracker Red CMXRos; CAS no. 167095-09-2) and JC1 (CAS no. 47729-63-5) as well as the nucleic acid stains SYTO 9 (SYTO 9 green fluorescent nucleic acid stain; the structure is proprietary), Hoechst 33342 (CAS no. 23491-52-3), and acridine orange (CAS no. 65-61-2) were also from Invitrogen. Daspei (CAS no. 3785-01-1) was obtained from Biotium, CFDA (CAS no. 79955-27-4), CFDA-SE (CAS no. 150347-59-4), SYBR green I (CAS no. 163795-75-3), and chloroquine diphosphate (CAS no. 50-63-5) (molecular weight [MW], 515.86) from Sigma, and methylene blue (CAS no. 61-73-4) (MW, 319.86) from AppliChem. Stock solutions were prepared according to the manufacturer's instructions. Further dilutions were prepared in complete culture medium. The chemical structures of the dyes are given in Fig. 1.

The antiplasmodial activity of the dyes and two reference drugs (chloroquine and methylene blue) were tested against two laboratory strains of *P. falciparum*, the chloroquine-sensitive 3D7 strain and the multiresistant Dd2 strain, by using the histidine-rich protein 2 (HRP2) assay as described previously (18). In brief, parasites were added at the ring stage to a 96-well plate in complete culture medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 50 μg/ml gentamicin, and 0.5% [wt/vol] AlbuMAX) at a parasitemia of 0.05% and a hematocrit of 1.5% and incubated for 3 or 6 days in the presence of a 3-fold serial dilution of the dyes. The 6-day assay was done to assess if the compounds exert their action in the subsequent cycle (delayed death) and was performed as described previously (12) with medium changes on day 2 and day 4 without replacement of the dye. After cultivation, plates were stored at −20°C until HRP2 measurement was performed with an enzyme-linked immunosorbent assay (ELISA). The assay was done in duplicate in at least three independent experiments for each dye.

The activity of MitoRed was additionally evaluated in clinical isolates from patients with malaria in Lambaréné, Gabon, between March and May 2009. Details of the study are described elsewhere (13). The experiments were approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné. The assay was performed as described for the laboratory strains except that parasites were cultivated at 37°C in a candle jar.

The 50% inhibitory concentration ($IC_{50}$) and $IC_{90}$ were determined by nonlinear regression analysis of log concentration-response curves using drc package v0.9.0 of R v2.6.1 (18a).

Of the 14 dyes tested, Hoechst 33342 and MitoRed showed the

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Address correspondence to Benjamin Mordmüller, benjamin.mordmueller@uni-tuebingen.de.
F. Joanny and J. Held contributed equally to this article.

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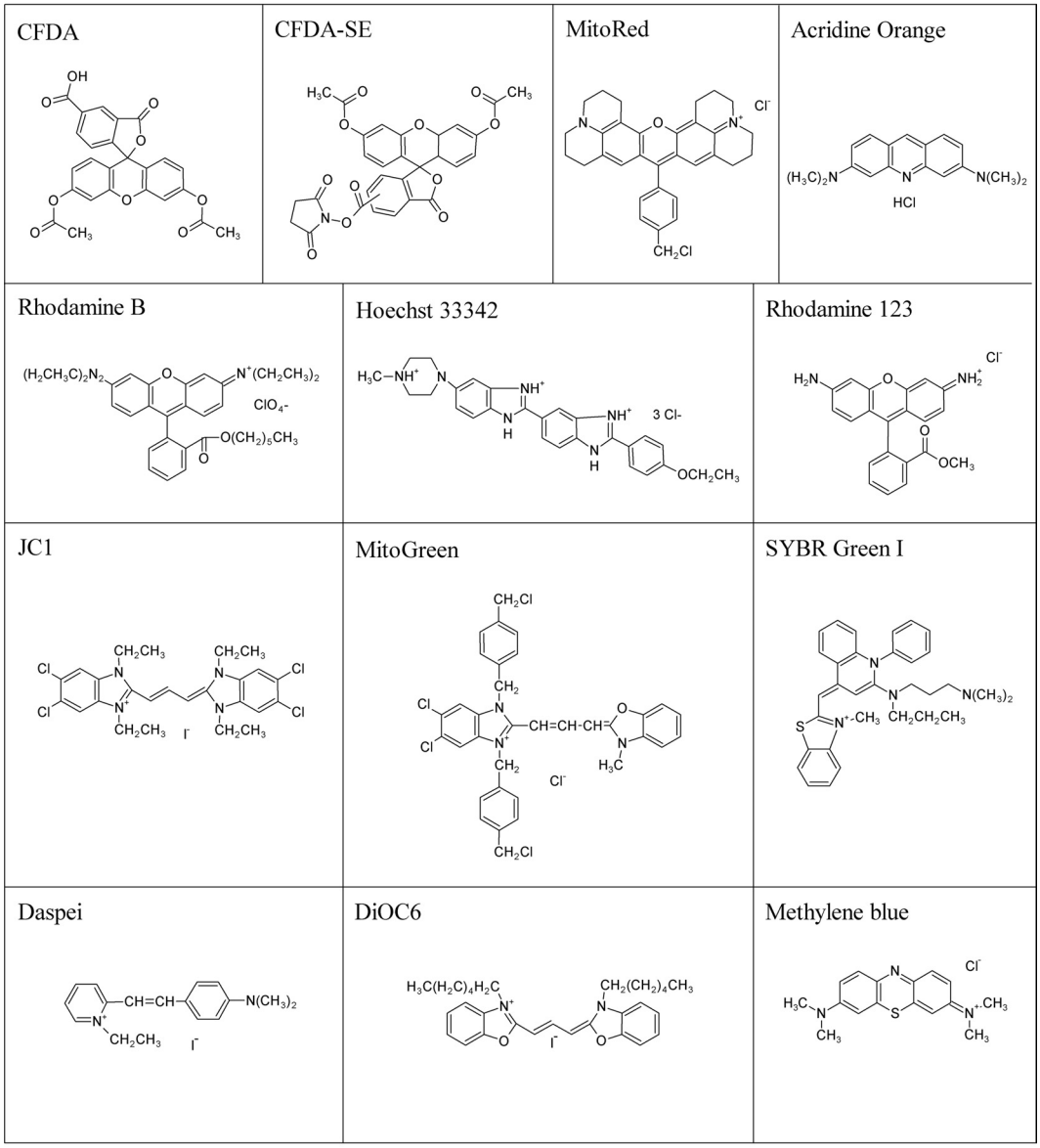


**FIG 1** Chemical structure of dyes used in the present study (the structures of SYTO 9 and SYTO 18 are proprietary).

highest antiplasmodial activity against both laboratory strains after 3 days (Table 1). MitoRed was also highly active against clinical isolates ($n = 19$) from Gabon ($IC_{50}$ geometric mean [range], 15.5 nM [3.4 to 35.9]; $IC_{90}$ geometric mean, 26.3 nM [13.3 to 59.6]). The levels of activity were similar against clinical and laboratory isolates, and $IC_{50}$s fell within a narrow range for the genetically diverse clinical isolates from Gabon, which is an area of high endemicity for *P. falciparum* malaria with high-grade chloroquine resistance (2). $DiOC_6$, SYTO 9, and rhodamine B showed high activities as well, with $IC_{50}$s similar to those of chloroquine and methylene blue (Table 1). Most stains were active in a low nanomolar concentration against the two laboratory strains. Delayed death, a typical feature of some antibiotics, which is characterized by cell death one cycle after drug exposure due to specific action on cell organelles (corresponding to 6 days in our assay), was not observed for the tested dyes. Indeed, some drugs had even lower activity after 6 days. This may be explained by the replacement of medium after 2 days without the replacement of the dye. Thus, if the dye does not have a delayed death effect, the surviving parasites can recover, leading to a higher $IC_{50}$. JC1, MitoGreen, rhodamine 123, acridine orange, and Daspei showed moderate antiplasmodial activity against both 3D7 and Dd2. SYTO 18 and SYBR green I had only low antiplasmodial activity. CFDA and CFDA-SE had no measurable antiparasitic activity at the highest concentrations tested (333 μM and 55 μM, respectively), showing that *P. falciparum* is not sensitive to their effects.

An ideal drug should act specifically against the parasite and not against the human host (or cell). Therefore, we evaluated the HeLa cell cytotoxicity of the dyes with the highest antiplasmodial activities using the neutral red assay (1). Each dye was tested at least three times.

To assess the safety of a compound, a selectivity index (SI) was

**TABLE 1** $IC_{50}$ and $IC_{90}$ of selected dyes and chloroquine against the culture-adapted strain of *P. falciparum* 3D7 or Dd2[a]

| Dye and *P. falciparum* strain | $IC_{50}$ ± SD (nM) | | $IC_{90}$ ± SD (nM) | |
|---|---|---|---|---|
| | Day 3 | Day 6 | Day 3 | Day 6 |
| Hoechst 33342 | | | | |
| 3D7 | 7.2 ± 3.1 | 18.9 ± 11.9 | 14.0 ± 8.8 | 25.8 ± 17.3 |
| Dd2 | 16.3 ± 11.2 | 28.0 ± 2.6 | 72.7 ± 107.9 | 36.5 ± 0.8 |
| MitoRed | | | | |
| 3D7 | 8.2 ± 9.6 | 10.6 ± 6.1 | 14.1 ± 19.5 | 15.3 ± 8.4 |
| Dd2 | 15.9 ± 10.9 | 6.3 ± 7.3 | 44.8 ± 24.0 | 9.0 ± 0.2 |
| $DiOC_6$ | | | | |
| 3D7 | 11.4 ± 8.1 | 17.3 ± 17.1 | 17.4 ± 9.4 | 24.1 ± 23.9 |
| Dd2 | 20.8 ± 12.2 | 168.3 ± 301.8 | 55.7 ± 31.5 | 229.0 ± 401.4 |
| Rhodamine B | | | | |
| 3D7 | 20.6 ± 12.6 | 19.2 ± 10.8 | 40.7 ± 12.4 | 29.5 ± 8.9 |
| Dd2 | 26.1 ± 15.3 | 69.5 ± 113.2 | 101.1 ± 34.6 | 117.2 ± 194.8 |
| SYTO 9 | | | | |
| 3D7 | 20.5 ± 20.0 | 23.1 ± 44.9 | 101.5 ± 105.7 | 41.0 ± 64.2 |
| Dd2 | 36.1 ± 17.7 | 4.9 ± 0.73 | 261.7 ± 114.1 | 10.8 ± 3.9 |
| JC1 | | | | |
| 3D7 | 107.8 ± 66.3 | 177.0 ± 55.0 | 170.6 ± 84.8 | 279.8 ± 95.36 |
| Dd2 | 100.1 ± 96.9 | 121.0 ± 128.2 | 269.9 ± 252.2 | 162.0 ± 156.3 |
| MitoGreen | | | | |
| 3D7 | 115.5 ± 149.9 | 187.1 ± 197.2 | 331.9 ± 407.5 | 245.2 ± 215.0 |
| Dd2 | 148.9 ± 102.7 | 103.8 ± 58.0 | 1,521 ± 2,335 | 150.8 ± 87.9 |
| Rhodamine 123 | | | | |
| 3D7 | 387.7 ± 208.6 | 189.2 ± 235.1 | 870.3 ± 567.6 | 490.3 ± 584.0 |
| Dd2 | 140.7 ± 117.4 | 287.2 ± 316.0 | 2,335 ± 3,893 | 414.2 ± 450.7 |
| Acridine orange | | | | |
| 3D7 | 465.7 ± 274.9 | 691.9 ± 391.8 | 1,772 ± 1,057 | 691.9 ± 391.8 |
| Dd2 | 166.0 ± 75.9 | 208.2 ± 22.5 | 620.4 ± 379.6 | 278.8 ± 31.4 |
| Daspei | | | | |
| 3D7 | 327.2 ± 327.2 | 502.7 ± 797.2 | 2,026 ± 2,364 | 834.7 ± 1298 |
| Dd2 | 591.3 ± 405.8 | 270.6 ± 267.2 | 55,046 ± 79,810 | 831.3 ± 1315 |
| SYTO 18 | | | | |
| 3D7 | 1,751 ± 969.8 | 873.1 ± 482.1 | 5,016 ± 3,186 | 1,916 ± 1,144 |
| Dd2 | 387.1 ± 207.6 | 636.3 ± 413.5 | 1,865 ± 381.1 | 1,141 ± 880.2 |
| SYBR green I | | | | |
| 3D7 | 2,585 ± 4,693 | 11,497 ± 17,785 | 4,669 ± 8,532 | 17,897 ± 28,028 |
| Dd2 | 2,100 ± 1,024 | 1,218 ± 583.2 | 14,062 ± 17,391 | 1,792 ± 872.5 |
| CFDA-SE | | | | |
| 3D7 | >55 μM | >55 μM | >55 μM | >55 μM |
| Dd2 | >55 μM | >55 μM | >55 μM | >55 μM |
| CFDA | | | | |
| 3D7 | >333 μM | >333 μM | >333 μM | >333 μM |
| Dd2 | >333 μM | >333 μM | >333 μM | >333 μM |
| Methylene blue | | | | |
| 3D7 | 7.8 ± 3.2 | 14.3 ± 10.7 | 13.3 ± 1.5 | 20.3 ± 13.4 |
| Dd2 | 14.3 ± 4.1 | 17.2 ± 12.4 | 29.2 ± 7.8 | 27.3 ± 20.2 |
| Chloroquine | | | | |
| 3D7 | 4.1 ± 1.8 | 6.2 ± 1.8 | 6.8 ± 3.3 | 7.8 ± 2.1 |
| Dd2 | 63.8 ± 30.1 | 84.6 ± 35.6 | 99.3 ± 45.3 | 131.8 ± 33.6 |

[a] Each value represents the geometric mean IC determined from the results of at least 3 independent experiments. Data in columns 2 to 5 represent nanomoles except where otherwise indicated.

**TABLE 2** Geometric mean determined from the results of at least 3 independent experiments for the 50% inhibitory concentration ($IC_{50}$) of the five dyes presenting the best antiplasmodial activity against human HeLa cells

| Dye | Cytotoxicity $IC_{50}$ ± SD (nM) on HeLa cells | SI[a] | | | |
|---|---|---|---|---|---|
| | | HC/3D7 (day 3) | HC/3D7 (day 6) | HC/Dd2 (day 3) | HC/Dd2 (day 6) |
| Hoechst 33342 | 1,357 ± 184.8 | 188 | 72 | 83 | 48 |
| MitoRed | 2,666 ± 1,238 | 325 | 251 | 168 | 423 |
| $DiOC_6$ | 2,727 ± 1,831 | 239 | 158 | 131 | 16 |
| SYTO 9 | 3,287 ± 1,141 | 160 | 142 | 91 | 671 |
| Rhodamine B | 4,372 ± 710.0 | 212 | 228 | 168 | 63 |

[a] SI, selectivity index (calculated as the ratio of the $IC_{50}$ against HeLa cells [HC] to the $IC_{50}$ against culture-adapted strains of *P. falciparum* 3D7 and Dd2).

calculated as the fractional ratio between the $IC_{50}$s for HeLa cells and *P. falciparum*. All five compounds with strong activity had high selectivity indices (Table 2), indicating that they are at least 50 times more toxic for *P. falciparum* than for HeLa cells. MitoRed had the highest selectivity, with indices between 168 and 423.

In this report, we demonstrate the high *in vitro* antiplasmodial activity of three mitochondrial stains, MitoRed, $DiOC_6$, and rhodamine B, and two nucleic acid stains, Hoechst 33342 and SYTO 9. MitoRed, like MitoGreen, rhodamine B, and rhodamine 123, is a rhodamine-based dye (Fig. 1). The activity of rhodamine dyes against plasmodia has already been tested in previous studies, but this is the first report of antiplasmodial activity of MitoRed and MitoGreen (14). In contrast to other reports (14), we found that rhodamine B was more active than rhodamine 123 against 3D7 and Dd2 after 3 days. This could be explained by the fact that we used the hexyl ester of rhodamine B, rendering the molecule positively charged and able to accumulate specifically in the mitochondrion (11), in contrast to other studies using the neutral molecule of rhodamine B (15).

$DiOC_6$ is a lipophilic green fluorescent dye that accumulates in mitochondria of living cells and is also able to stain the endoplasmic reticulum when used at higher concentrations (>1 μM). It was previously shown that it can reversibly impair mitochondrion functions in yeast (16), which could explain the higher $IC_{50}$ in the 6-day experiment performed with Dd2. Indeed, in the 6-day experiment, a fraction of the drug was removed after 2 days of incubation, allowing the recovery and growth of the parasites until day 6.

The two other dyes with strong antiplasmodial activity were Hoechst 33342 and SYTO 9, both able to stain nucleic acids. Hoechst 33342 did not exhibit intercalation while staining double-stranded DNA with a preference for AT-rich regions. The rather high selectivity index may be explained by the higher AT content of *P. falciparum* compared to human DNA. Hoechst 33258, a related dye, was previously shown to be antiparasitic as well (8). Hoechst 33342 contains an additional ethyl group, rendering it more lipophilic and thus more membrane permeable than Hoechst 33258, which may explain the better antiplasmodial activity of Hoechst 33342. Since Hoechst 33342 was previously shown to inhibit topoisomerase I (3) and increase the mutation rate (5), it is not a preferred candidate for clinical development.

However, this pilot experiment shows that prescreening for staining properties may be an interesting way to build highly efficient pathogen-specific compound libraries. It is estimated that currently used antimalarials belong to only 10 chemotypes (9).

Simply by screening for staining properties, we showed the antimalarial activity of five molecules which do not belong to these chemotypes and thus could lead to the development of new classes of antimalarial molecules that are not hampered by preexisting resistance mechanisms (6). A next step could be the testing of these dyes for *in vivo* activity and toxicity in mouse models. The most promising candidate from our experiments is MitoRed, as it showed high activity against laboratory strains and clinical isolates and low toxicity against HeLa cells. Since some rhodamine derivatives are even used in cosmetics and have little toxicity in humans (4), further development may be interesting.

JC1, MitoGreen, rhodamine 123, and Daspei presented lower antiplasmodial activity and are therefore unlikely to be further developed as antimalarial drugs. SYTO 18, acridine orange, SYBR green I, CFDA, and CFDA-SE showed very low or no antiplasmodial activity compared to chloroquine or methylene blue. This observation may have interesting implications for the development of new methods to track parasites with minimal interference with their viability.

In conclusion, five dyes, Hoechst 33342, MitoRed, $DiOC_6$, SYTO 9, and rhodamine B, show high antiplasmodial activity and low cytotoxicity against human HeLa cells and could be further developed as antimalarial drugs. On the other side of the spectrum, dyes with low toxicity against *P. falciparum* can be used to stain parasites without interfering with cell growth or metabolism.

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